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13. ABSTRACT *(Maximum 200 Words)*

The etiology of Parkinson's disease is not known and may be related to several factors which include inheritable mutations (genetic), exposure to environmental toxins, and/or traumatic head injury. Our current research examines age-related changes in neurotrophic factor expression in Brown Norway/(Fischer 344 F1 hybrid (F344BNF$_1$)rats, and we have preliminary evidence that the young and aged nigrostriatal system responds differently to neurotoxic insult or mechanical injury, *i.e.*, young rats show a tendency to increase neurotrophic factor expression while aged rats do not. This is an important finding in the sense that the success of new therapies utilizing embryonic neurons or stem cells may be dependent on how well the implanted cells interact with the host neurotrophic environment. The studies proposed in this research project will further characterize the temporal expression of neurotrophic markers before and after neurotoxic insult or mechanical injury to the nigrostriatal system in young, middle-age, and old F344BNF$_1$ rats. The second part of this project will demonstrate that age differences in compensatory neurotrophic mechanisms that occur in the nigrostriatal system have a direct impact on the success of embryonic neurons implanted into the injured or denervated striatum.

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Introduction

The main hypothesis to be tested is whether or not molecular markers for neurotrophic factors and their receptors show a greater compensatory response to neurotoxic insult or injury in young brain than in older brain in the nigrostriatal system. Our preliminary studies indicate that the aged brain does not increase the expression of several neurotrophic factors markers indigenous to nigrostriatal system following degenerative lesions. This is important from the standpoint that strategies which employ living cells to restore or release factors beneficial to injured brain regions may also require supplemental neurotrophic support for implanted cells, particularly if trophic mechanisms are diminished in the aging brain. We will test this hypothesis by implanting fetal dopaminergic grafts into the brains of young, middle-age, or old rats at various times relative to the lesion or injury, and then assess the integrity of the grafts. Based upon our preliminary studies, we hypothesize that grafts placed into the brains of young rats will show better graft survival and function than grafts placed into older brain. And lastly, we hypothesize that graft survival and function in aged rats with nigrostriatal injuries can be improved with supplemental treatments of neurotrophic factors.

Body

Experiments in year 1 were a continuation of the preliminary studies that showed age-related changes in neurotrophic factor expression following a neurotoxic lesion of the nigrostriatal pathway. Studies in year 1 focused on temporal changes of neurotrophic factor protein expression relative to the time of the neurotoxic lesion of nigrostriatal pathway in young adult brain. Due to a shortage of aged F344BNF$_1$ rats at NIA Aging colony when the first year project began, we had to purchase younger rats and allow them to age in our animal facility during the first year. These animals only recently reached an appropriate age for our studies; therefore most of the data reported in the first year progress report were obtained from young adult rats.

In young adult rats with a neurotoxin lesion of the nigrostriatal pathway, we observe a transient change in the expression of two endogenous neurotrophic factors: brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF). Both neurotrophic factors are elevated in the denervated striatum 2-4 weeks following the lesion, however, by the 12th post-lesion week both neurotrophic factors return to levels similar to those found in the intact striatum[10] (see appendix 1 for review). Interestingly, during the first week post-lesion we observe a significant drop of BDNF protein expression in the denervated striatum while GDNF protein in the denervated striatum is not significantly different than that measured in the intact striatum of young adult rats.

Transplants of fetal dopaminergic neurons implanted into the denervated striatum at several different post-lesion time points show that after 1 week post-lesion or 4 weeks post-lesion, dopamine grafts exhibit the best survival and functional reinnervation than grafts implanted immediately following the lesion or when implantation is delayed until the 12th post-lesion week. Moreover, the survival and fiber outgrowth of transplanted fetal dopamine neurons correlated well with the concomitant changes in BDNF and GDNF protein expression within the denervated striatum of young adult rats.

In situ hybridization studies yielded several interesting age-related findings for the expression of dopaminergic or neurotrophic factor markers. For instance, we observed a significant age-related decline in the expression of tyrosine hydroxylase (TH) mRNA in

the ventral midbrain. We also observed that the expression of erbB4 receptor mRNA showed a similar age-related decline; erbB4 receptor binds neuregulin and the neuregulins have been shown to exert neurotrophic support for dopaminergic neurons. Whether or not the decline in TH or erbB4 mRNA expression is directly related to an age-related loss of dopaminergic neurons has yet to be determined because of the numerous conflicting reports of age-related changes in dopaminergic neuron survival and function during the normal aging process[1-7, 9]. On the other hand, there does not appear to be significant age-related changes in the expression of mRNAs for other neurotrophic factor markers including BDNF, NT-3, trkB, or trkC during the normal aging process of the nigrostriatal system.

During the first year we attempted to produce a traumatic lesion of the nigrostriatal pathway; this was proposed as an alternate method for lesioning the nigrostriatal pathway. We attempted to induce a traumatic lesion by using a Scouten knife to sever the fibers of the medial forebrain bundle (MFB); this bundle contains dopaminergic fibers projecting from the ventral midbrain to forebrain target sites including the striatum. After making numerous adjustments to both the lesion coordinates and the size of the knife cut, we were unable to consistently generate a lesion comparable to that produced using the 6-hydroxydopamine (6-OHDA) neurotoxic lesion method. The traumatic lesions showed very little loss of either dopaminergic terminals in the striatum or dopaminergic cells in the ventral midbrain despite histological verification of accurate knife cuts. We have not completely abandoned our attempts at traumatic lesions, however. Our next set of experiments will use Scouten knife cuts as a means to disrupt the dopaminergic terminal fields and observe the consequential changes in neurotrophic factor expression[8].

Key Research Accomplishments

- Expression of BDNF and GDNF protein in the denervated striatum is elevated transiently following a neurotoxic lesion of the nigrostriatal pathway in young but not old brain.

- Transplants of fetal dopaminergic neurons show better survival and functional reinnervation at the same post-lesion time points GDNF and BDNF are elevated in the denervated striatum of young adult rats.

- Age-related declines in the expression of TH and erbB4 receptors mRNAs are observed in the nigrostriatal system, but BDNF, NT-3, trkB, or trkC mRNAs remain unchanged.

Reportable Outcomes

The research published in the manuscript below was partly supported by this grant, however, it was not cited in the acknowledgements because of an accidental omission:

Yurek DM, and Fletcher-Turner A (2002) Temporal changes in the neurotrophic environment of the denervated striatum as determined by the survival and outgrowth of grafted fetal dopamine neurons, *Brain Res.* **931**:126-134.

Conclusions

Results from year 1 experiments support the hypothesis neurotrophic factors are transiently elevated in components of the basal ganglia following a neurotoxic lesion of the nigrostriatal pathway of young adult rats. We also observed that this compensatory response to a neurotoxic lesion is lacking or diminished in the aged rat brain. Furthermore, the compensatory response observed in young adult rats appears to directly affect the outcome of cellular replacements therapies. Fetal dopamine neurons implanted into the denervated striatum of young adult rats show better survival and more robust fiber outgrowth into the host brain at the same time two endogenous neurotrophic factors, BDNF and GDNF, are elevated in the denervated striatum. Studies just being and continuing into the second year of this project will determine whether or not transplants of fetal dopamine neurons are affected by the diminished neurotrophic factor compensatory response that occurs in the aged rat brain following a neurotoxic lesion. We also plan to modify the traumatic lesion model so that a more severe, and more consistent, lesion of the nigrostriatal can be used in these studies.

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Appendices

1. Yurek DM, Fletcher-Turner A (2002) Temporal changes in the neurotrophic environment of the denervated striatum as determined by the survival and outgrowth of grafted fetal dopamine neurons, *Brain Res.* **931**:126-134.

2. Yurek DM, Fletcher-Turner A (2001) Differential expression of GDNF, BDNF, and NT-3 in the aging nigrostriatal system following a neurotoxic lesion, *Brain Res.* **891**:228-235.

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Research report

Temporal changes in the neurotrophic environment of the denervated striatum as determined by the survival and outgrowth of grafted fetal dopamine neurons

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Abstract

There is growing evidence that the neurotrophic environment of the denervated striatum may change with time following a lesion of the nigrostriatal pathway in young adult rats. To test this hypothesis, we implanted fetal dopamine grafts into the striatum at several different time points relative to the nigrostriatal pathway lesion and allowed the grafts to integrate with the host for a period of 1 month; subsequently, we observed the function and morphology of the dopamine grafts. Fetal grafts were implanted at the following time points relative to the lesion: 1 week before (−1 Week), at the same time (Week 0), 1 week after (1 Week), 4 weeks after (4 Weeks), or 12 weeks after (12 Weeks). Amphetamine-induced rotational behavior was assessed 4 weeks after grafting for all groups. Rotational scores indicate that grafts for the 1 Week group showed the greatest reversal of amphetamine-induced rotational behavior that was also significantly greater than the scores for the −1 Week group. Morphological analysis revealed that grafts in the Week 0, 1 Week and 4 Weeks groups showed a significantly larger area of tyrosine hydroxylase-positive (TH+) fiber outgrowth than in the −1 Week group, while fiber outgrowth for the 12 Weeks group was significantly lower than for the 1 Week group. Cell count analysis for TH+ neurons within the graft indicate a significantly greater number of TH+ neurons in grafts for the 1 Week group than in grafts for the −1 Week. The results of this study suggest that neurotoxic lesions may induce a compensatory increase in neurotrophic activity within the denervated striatum of young rats that is conducive to the survival and outgrowth of fetal dopamine grafts. These data also correlate well with reports that the expression of several specific dopaminergic neurotrophic factors within the striatum increase following a neurotoxic lesion of the nigrostriatal pathway in young adult rats.

Theme: Development and regeneration

Topic: Transplantation

Keywords: Dopamine; Neural transplantation; Parkinson's disease; Neurotrophic factor; Glial cell line-derived neurotrophic factor; Brain-derived neurotrophic factor; 6-Hydroxydopamine; Rodent; Striatum

1. Introduction

Several studies have provided evidence that neurotoxic and ablative lesions of the nigrostriatal pathway induce an increase in neurotrophic activity within the denervated striatum. Chadi et al. [7] demonstrated an immediate increase of basic fibroblast growth factor (bFGF) mRNA and immunoreactivity in the denervated striatum following a 6-hydroxydopamine (6-OHDA) lesion of the nigrostriatal pathway. Specific neurotrophic factors, e.g. brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), increase expression within the denervated striatum following a 6-OHDA lesion in young adult brain [32,33,39]. The increase in striatal neurotrophic activity following a nigrostriatal pathway lesion is further substantiated by evidence that striatal extracts taken from the denervated striatum enhance the survival of cultured dopamine neurons [5,25]. The specific neurotrophic factors that increase their expression in the denervated striatum, e.g. bFGF, BDNF, and GDNF, have

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been shown to provide potent neurotrophic support to dopamine neurons in vitro [12,14,17,18,20,22,24,38]. The increased expression of neurotrophic factors within the denervated striatum may be an underlying mechanism that supports differentiation, survival, and functional outgrowth of grafted embryonic neurons. Previous studies have shown that fetal dopamine grafts supplemented with neurotrophic factors can successfully improve the survival and function of the grafts [3]; in particular, treatment of fetal dopamine grafts with exogenous BDNF and GDNF before or after implantation of the grafts improves the function and survival [1,26–28,31,34,37].

The purpose of the present study was to compare the survival, fiber outgrowth, and function of fetal dopamine grafts when these grafts are implanted into the lesioned or intact striatum of young adult rats, and determine whether lesioned-induced neurotrophic activity may be beneficial to graft development and function.

2. Material and methods

2.1. Animals

A total of 54 young (4–5 months old) male Sprague–Dawley rats were obtained from Harlan Farms and used in this study. Animals were housed in environmentally regulated rooms and had free access to food and water for the duration of the study. All animal procedures were conducted in strict compliance with approved institutional protocols, and in accordance with the provisions for animal care and use described in the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, 1985).

2.2. Ventral mesencephalic tissue grafts

Recipient animals were anesthetized with halothane (1.0–1.5% mixture with air) and placed in a stereotaxic apparatus. At the same time, the ventral mesencephalon was dissected from E14 fetuses obtained from time-pregnant Sprague–Dawley rats (Harlan Farms) and stored individually in a cold, sterile, calcium-magnesium free buffer (CMF: 0.15 M NaCl, 8.0 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM $KHPO_4$, 26.0 mM $NaHCO_3$, 0.1% glucose, 100 mg/ml streptomycin, 2.5 mg/ml fungizone, pH 7.2). The ventral mesencephalon from a single fetus was drawn into the blunt end of a 22-gauge spinal needle and stereotaxically placed into the denervated striatum of the recipient animal at the following coordinates: AP +0.5, ML +2.5, DV −5.5. Animals received grafts according to the following schedule: for the −1 Week group (n=8), grafts were implanted into the intact striatum 1 week before the ipsilateral nigrostriatal pathway was lesioned; for the Week 0 group (n=6), each animal received a unilateral 6-OHDA lesion and immediately thereafter a graft was implanted into the ipsilateral striatum; for the 1 Week (n=9), 4 Weeks (n=8), and 12 Weeks (n=6) groups, grafts were placed into the lesioned striatum 1, 4, or 12 weeks after the 6-OHDA lesion, respectively.

2.3. 6-Hydroxydopamine lesions

All rats were given unilateral 6-hydroxydopamine (6-OHDA) lesions of the nigrostriatal pathway; 6-OHDA (Sigma) was dissolved in 0.9% saline (containing 0.2% ascorbic acid) at a concentration of 3.0 μg/μl and stereotaxically injected into the nigrostriatal pathway of anesthetized rats at a rate of 1.0 μl/min for 2 min. Each rat received two injections of 6-OHDA: one in the vicinity of the medial forebrain bundle (AP −4.4, ML 1.2, DV −7.5) and the other in the rostral pars compacta of the substantia nigra (AP −5.3, ML 2.0, DV −7.5); all coordinates reported in this study represent millimeter adjustments from bregma (AP, ML) and below the dural surface (DV) with the top of the skull in a flat position. This technique routinely produces complete lesions of dopamine neurons in the A9 and A10 midbrain regions, and near complete denervation of dopaminergic fibers innervating the ipsilateral striatum.

2.4. Quantification of neurotrophic factors by an enzyme-linked immunosorbent assay (ELISA)

A total of 17 rats were euthanatized either 3 days (n=10) or 12 weeks (n=7) after receiving a unilateral 6-OHDA lesion. Brains were removed, the striatal and ventral midbrain brain regions of both hemispheres were dissected on ice and the samples were then stored at −80 °C. Subsequently, each tissue sample was homogenized in 300-μl volumes of homogenate buffer (400 mM NaCl, 0.1% Triton-X, 2.0 mM EDTA, 0.1 mM benzethonium chloride, 2.0 mM benzamidine, 0.1 mM PMSF, Aprotinin (9.7 TIU/ml), 0.5% BSA, 0.1 M phosphate buffer, pH 7.4). The homogenate was centrifuged for 10 min at 10,000×g at 4 °C. The homogenate was divided into 100-μl duplicate samples and neurotrophic factor content was determined using an antibody sandwich format: extracted neurotrophic factors from each sample were captured with a monoclonal antibody against BDNF or GDNF and the captured neurotrophic factor was then bound to a second, specific, polyclonal antibody (pAb) against BDNF or GDNF. After washing, the amount of specifically bound pAb was detected using a species-specific anti-IgY antibody conjugated to horseradish peroxidase (HRP) as a tertiary reactant. Unbound conjugate was removed by washing and, following an incubation period with a chromogenic substrate, the color change was measured in a microplate reader (450 nm). The amount of neurotrophic factor was proportional to the color change generated in an oxidation–reduction reaction; the Promega E_{max}™ ImmunoAssay System was used for the detection of both neurotrophic factors. The reliability of the neuro-

trophic factor measures ranged from 97 to 99% based upon regression analysis. We chose not to examine the expression of BDNF or GDNF at time points between 3 days and 12 weeks post-lesion because these studies were performed earlier [32,33].

2.5. Rotational behavior

Amphetamine-induced rotational behavior was tested in all treatment groups 4 weeks after grafting. Rotational behavior was induced by a systemic injection of amphetamine (5.0 mg/kg, i.p.). Rats were placed inside opaque 16-inch diameter cylindrical chambers which were positioned directly beneath a video camera. The video camera was connected to a Videomex V image motion computer system (Columbus Instruments, Columbus, OH). The total number of 360° clockwise or counterclockwise rotations was measured during each 90-min test session. No post-lesion, pre-graft rotational scores are reported because three of the five treatment groups received fetal grafts at time points before the 6-OHDA lesions were fully developed.

2.6. Immunohistochemical technique

Rats were sacrificed at the end of the 6th postgraft week for all treatment groups. All rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with ice-cold saline followed by 4% paraformaldehyde. The brains were post-fixed overnight in 4% paraformaldehyde and placed in 30% sucrose. Brain sections (40 μm) were cut on a sliding microtome and stored in cryoprotectant at −20 °C [30]. For immunohistochemical detection of tyrosine hydroxylase (TH) free-floating sections were incubated overnight in mouse antisera containing a monoclonal antibody against TH (1:8000; Chemicon). The sections were then incubated in an affinity-purified biotinylated goat anti-mouse IgG secondary antibody (1:800, Chemicon, Temecula, CA) and then incubated in an avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA). Staining was completed by placing the sections in 0.003% H_2O_2 that contained diaminobenzidine chromogen to visualize the peroxidase-catalyzed reaction product. To enhance fiber staining, nickel ammonium sulfate was added to the last step.

2.7. Cell counts and quantification of fiber outgrowth

Cell counts were made using light microscopy. Counts of TH+ cell bodies were made in every third section throughout the rostral-caudal extent of the lesioned/transplanted striatum. Particles less than 5.0 μm were not counted. The total number of TH+ cell bodies was summed for each animal and an average value (±S.E.M.) was calculated for each of the three different treatment groups. Cell counts were made with the observer blind to the treatment.

Fiber outgrowth from transplants was quantified using methodology from a previous study [35]. Briefly, low power (2×) images of brain sections containing TH immunostained transplants were captured via a video frame grabber and stored to computer disk as TIFF files; approximately six to eight brain sections containing grafts were used for analyses. Image files were analyzed on a Macintosh IIsi computer using the public domain NIH Image program. Coarse fibers, cell bodies, and fine granules immunostained for TH were distinguished from one another by their detection at different density levels. For example, fine TH-ir elements distributed diffusely within the host striatum were measured by adjusting density levels to exclude TH+ cell bodies and background from the calculation. All density measurements were made with the observer blind to the treatment.

2.8. Statistical analysis

Analysis of variance (ANOVA) was used to analyze the effect of treatment (transplantation time relative to lesion) on the dependent variables: rotational scores, cell counts, and area of fiber outgrowth. Results for the ELISA analysis were analyzed using ANOVA. Student-Newman-Keuls was used for post hoc mean comparisons for all ANOVAs showing a significant treatment effect. The alpha level was set to 0.05.

3. Results

3.1. Post-lesion measurements of BDNF or GDNF protein in striatum or ventral midbrain

Table 1 summarizes BDNF and GDNF protein in the striatum or ventral midbrain immediately after (3 days) or 12 weeks after naïve rats received unilateral 6-OHDA lesions. Levels of BDNF and GDNF protein are greater in the lesioned ventral midbrain than in the intact side 3 days after a 6-OHDA lesion. At this same time point, we do not

Table 1
Measurement of BDNF or GDNF 3 days or 12 weeks post-lesion (ng/g tissue)

Brain region	BDNF		GDNF	
	3 days	12 weeks	3 days	12 weeks
Striatum				
Intact side	11.1±0.8	11.5±0.6	9.0±0.4	11.1±0.4
Lesioned side	10.1±0.9	11.1±1.0	8.8±0.6	14.3±1.4
Ventral midbrain				
Intact side	10.8±0.6	13.0±1.0	9.1±0.3	10.7±0.1^
Lesioned side	16.0±1.5*	15.7±1.5	11.0±0.7^^	8.7±1.0

*P=0.007 versus intact side. ^P=0.06 versus lesioned side (approached significance). ^^P=0.02 versus intact side.

Table 2
Relative changes of BDNF or GDNF at several post-lesion time points

	3 Days	2 Weeks*	4 Weeks^	12 Weeks
BDNF				
Lesioned striatum	n.d.	↑	↑	n.d.
Lesioned ventral midbrain	↑	↑	↑	n.d.
GDNF				
Lesioned striatum	n.d.	↑	n.r.	n.d.
Lesioned ventral midbrain	↑	n.d.	n.r.	↓

↑, significant increase relative to intact side. ↓, significant decrease relative to intact side. n.d., no difference. n.r., not reported. * Data initially reported in [33]; ^ data initially reported in [32].

observe significant differences in protein levels between the lesioned and intact striatum for either BDNF or GDNF. At 12 weeks post-lesion, BDNF and GDNF protein levels are the same in the intact and lesioned sides for both the striatum and ventral midbrain. The mean value of GDNF protein in the intact ventral midbrain is greater than that in lesioned ventral midbrain, however, the statistical comparison of GDNF of these two means only approaches significance ($P=0.06$). Table 2 summarizes changes in the expression of BDNF and GDNF protein levels in the nigrostriatal pathway at various post-lesion time points.

3.2. Rotational behavior

Statistical analysis of rotational scores revealed a significant effect of treatment ($F(4,36)=2.92$, $P=0.03$). In Fig. 1, lesioned animals receiving transplants in all five treatment groups show functional compensation as determined by the low rates of amphetamine-induced rotational behavior observed in these animals 4 weeks after grafting. Statistical comparison of rotational scores revealed significantly lower scores for the 1 Week group when compared to the −1 Week group.

3.3. Cell counts of transplanted TH+ neurons

Statistical analysis of cell count data revealed a significant effect of treatment ($F(4,36)=2.67$, $P=0.05$). The average number of TH+ neurons in transplants for the 1 Week group was more than double and statistically greater than the average number counted in the −1 Week group (Fig. 2).

3.4. Fiber outgrowth

Statistical analysis of fiber outgrowth revealed a significant effect of treatment ($F(4,36)=3.30$, $P=0.02$). Similar to the cell count data, animals in the 1 Week group showed an average area of TH+ fiber staining in the host tissue surrounding the transplant that was over double the area of TH+ fiber staining observed in the −1 Week group (Fig. 3). The area of TH+ fiber outgrowth was significantly greater for animals in the Week 0 or 4 Weeks groups than in the −1 Week group. Fig. 4 shows the four best examples of TH+ fiber staining in the lesioned/trans-

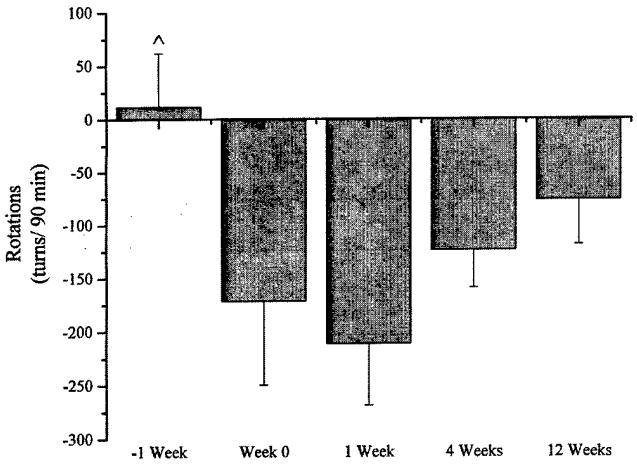

Fig. 1. Amphetamine-induced rotational scores for animals in each treatment group 4 weeks after grafting. Grafts were implanted at the following time points relative to the 6-OHDA lesion: 1 week before ($n=8$, −1 Week), at the same time, ($n=6$, Week 0), 1 week after ($n=9$, 1 Week), 4 weeks after ($n=8$, 4 Weeks), or 12 weeks after ($n=6$, 12 Weeks). Bars represent the average rotational score for each treatment group±S.E.M. Rotational behavior was induced with amphetamine (5.0 mg/kg, i.p.) and the total number of ipsilateral (positive) and contralateral (negative) rotations were counted over a 90-min post-injection period. Scores for the 1 Week group were significantly greater than the scores for −1 Week group. ×$P<0.05$, 1 Week versus −1 Week.

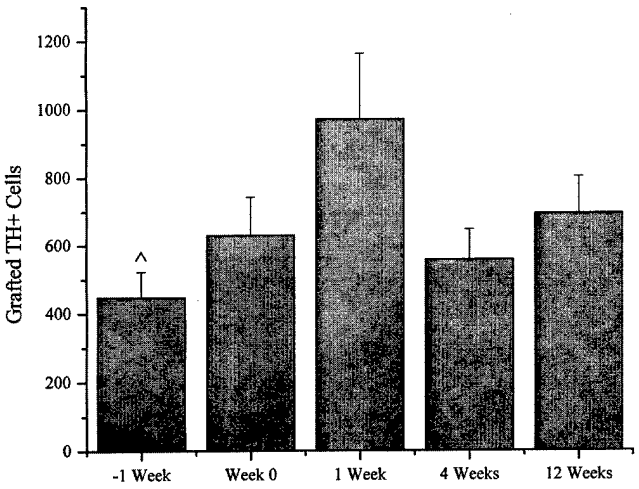

Fig. 2. Total number of TH+ cell bodies counted in dopamine grafts for each of the five treatment groups. Grafts were implanted at the following time points relative to the 6-OHDA lesion: 1 week before ($n=8$, −1 Week), at the same time, ($n=6$, Week 0), 1 week after ($n=9$, 1 Week), 4 weeks after ($n=8$, 4 Weeks), or 12 weeks after ($n=6$, 12 Weeks). Brains were sliced into 40-μm sections and immunohistochemically stained for TH. The total number of TH+ cell bodies was counted in every third section throughout the rostral-caudal extent of the lesioned/transplanted striatum. Bars represent an average of the total number of TH+ cell bodies for each animal in each treatment group±S.E.M. Cell counts for the 1 Week group were significantly greater than cell counts for the −1 Week group. ×$P<0.05$, 1 Week versus −1 Week.

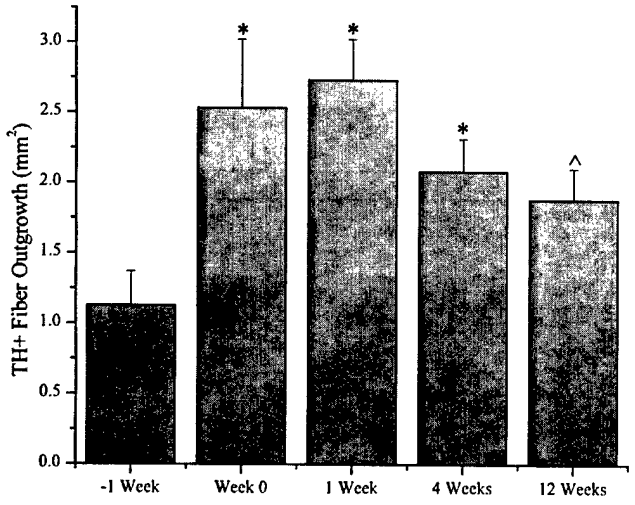

Fig. 3. Total area of TH+ fiber outgrowth from dopamine grafts for each treatment group 5 weeks after grafting. Grafts were implanted at the following time points relative to the 6-OHDA lesion: 1 week before (n=8, −1 Week), at the same time, (n=6, Week 0), 1 week after (n=9, 1 Week), 4 weeks after (n=8, 4 Weeks), or 12 weeks after (n=6, 12 Weeks). Brains were sliced into 40-μm sections and immunohistochemically stained for TH. Densitometry was set to detect TH+ fibers projecting from the graft and TH+ reinnervation of the host striatum; TH+ cell bodies and fibers within the transplant, as well as background, were excluded from the analysis. Area calculations were made in every third section throughout the rostral-caudal extent of the lesioned/transplanted striatum and an average area of TH+ fiber outgrowth was calculated for each animal. Bars represent an average area of fiber outgrowth for each treatment group±S.E.M. Fiber outgrowth for the 1 Week group was significantly greater than outgrowth for the −1 Week or 12 Week groups. *P<0.05 versus −1 Week, ×P<0.05 versus Week 1.

planted and intact striata for both the −1 Week and 1 Week groups.

3.5. Correlation of behavior and graft morphology

The behavioral and morphological scores presented above were pooled for all treatment groups and individual scores for rotational behavior were plotted as a function of the number of TH+ neurons in the graft (Fig. 5A) or as a function of the area of TH+ outgrowth from the graft (Fig. 5B). Regression analysis was performed on these scatter plots and our analysis revealed that the decrease in rotational scores following grafting is more tightly correlated with the extent of grafted fiber outgrowth (r^2=−0.71) than it is with the number of TH+ neurons within the graft (r^2=−0.35).

4. Discussion

The results of this study show that factors within the denervated striatum provide a more enriched environment than the intact striatum for graft development and function. Grafts placed into the denervated striatum within a 1-month period after the nigrostriatal pathway is lesioned show significantly better fiber outgrowth than grafts placed initially into an intact striatum. The survival of grafted dopamine neurons is also improved if the grafts are placed into the denervated striatum within 1 week after the nigrostriatal pathway lesion. These results suggest that in young rats, a neurotoxic lesion of the nigrostriatal pathway induces an increase of neurotrophic activity that is beneficial to the survival and functional outgrowth of fetal dopamine grafts. Moreover, this effect may be transient and dependent upon the length of time between the lesion and grafting procedures.

It is interesting that fiber outgrowth from fetal dopamine grafts is improved at the same post-lesion time points when specific dopaminergic neurotrophic factors are known to increase their expression in the lesioned striatum relative to the intact striatum. In previous studies we observed an improvement of fiber outgrowth from dopamine grafts when grafts were exposed to continuous infusion of exogenous BDNF during the 1st month after grafting [35] or for a 2-week infusion period that began at the end of the 2nd post-transplantation week [34]; Sauer et al. reported that BDNF infusions into dopamine grafts improve function without a concomitant increase in the number of surviving grafted dopamine neurons [27]. In young adult rats, we and others observe an increase in BDNF protein levels within the lesioned striatum that is apparent 2–4 weeks after the lesion [32,33,39]; not only is striatal BDNF elevated 2 weeks after a nigrostriatal pathway lesion, but so is another dopaminergic neurotrophic factor, GDNF. In this study we also measured BDNF and GDNF protein in animals with lesions only and did not observe an increase of either neurotrophic factor in the lesioned striatum immediately (3 days) or 12 weeks after the lesion. This finding, combined with the results from our earlier studies, suggests that neurotoxic lesions induce transient increases in neurotrophic factor expression in the striatum for a period of at least 1 month that may not begin immediately after the administration of the neurotoxin. Of all the treatment groups tested in this study, dopamine grafts implanted into the 1 Week group would most likely be exposed to elevated levels of endogenous BDNF and GDNF at the same time period when exogenous infusion of these neurotrophic factors improves the survival and fiber outgrowth of fetal dopamine grafts. The transient increase of neurotrophic factors within the lesioned striatum may be one explanation why the area of TH+ fiber outgrowth from grafts is higher when the grafts are implanted post-lesion rather than pre-lesion. Nonetheless, it is clear that removal of dopaminergic afferents to the striatum is a requirement to stimulate significant fiber outgrowth from fetal dopamine grafts.

The relatively poor fiber outgrowth and survival of grafted dopamine neurons observed in the −1 Week group could be attributable to several factors. While a lack of lesion-induced increase of neurotrophic activity may be one likely explanation for diminished fiber outgrowth, it is

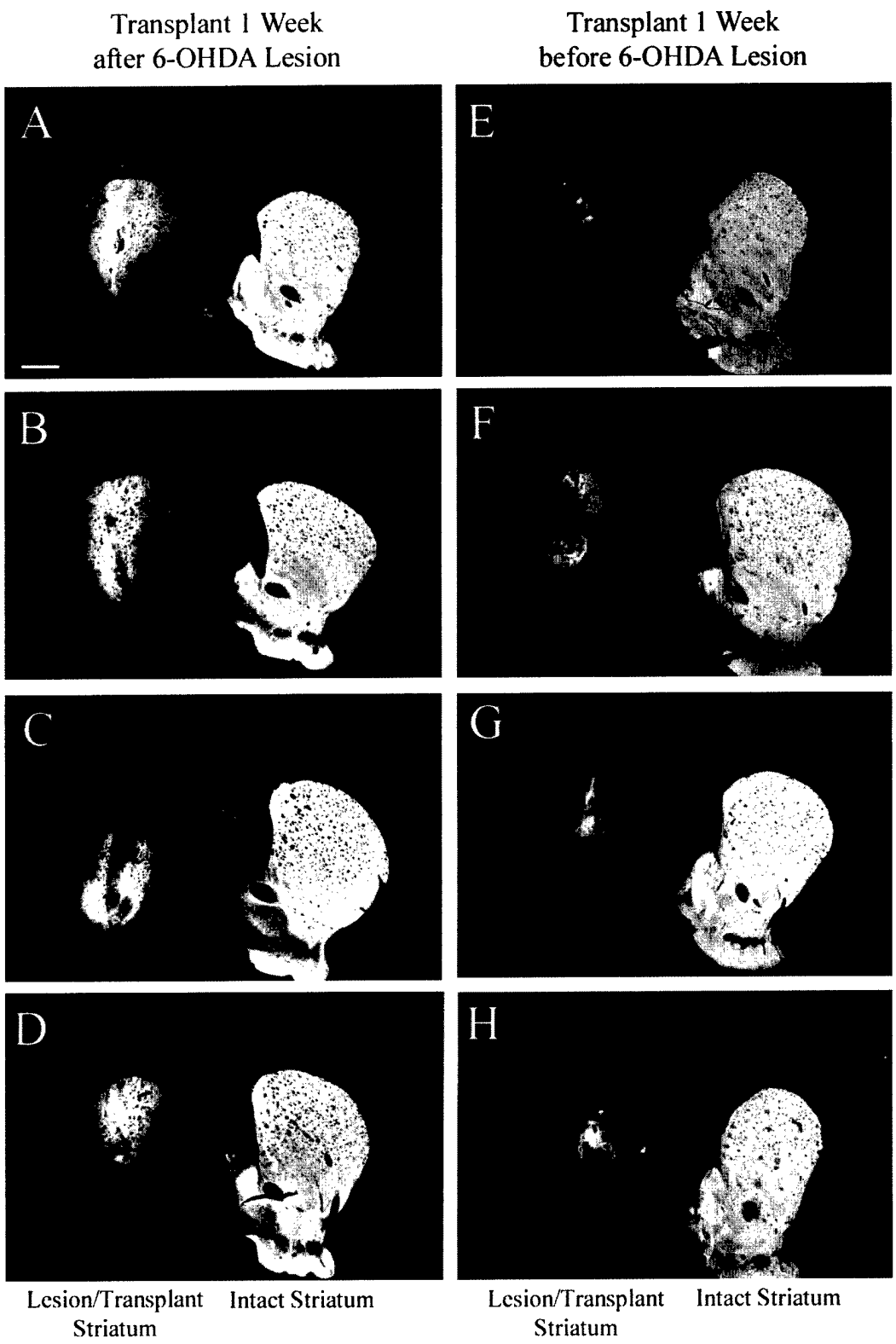

Fig. 4. Dark-field photomicrographs of coronal brain sections stained for TH and showing the four best examples of TH+ staining in the lesioned/transplanted striatum for the 1 Week (left column, panels A–D) and −1 Week (right column, panels E–H) treatment groups. For each panel, the left side of the brain is the lesioned/transplanted side and the right side is the intact side. Each panel is from a different animal. Note the larger area of TH+ fiber outgrowth in lesioned/transplanted striatum of the 1 Week group when compared to same region in the −1 Week group. Brain sections are 40 μm in thickness. Calibration bar in panel A: 1 mm.

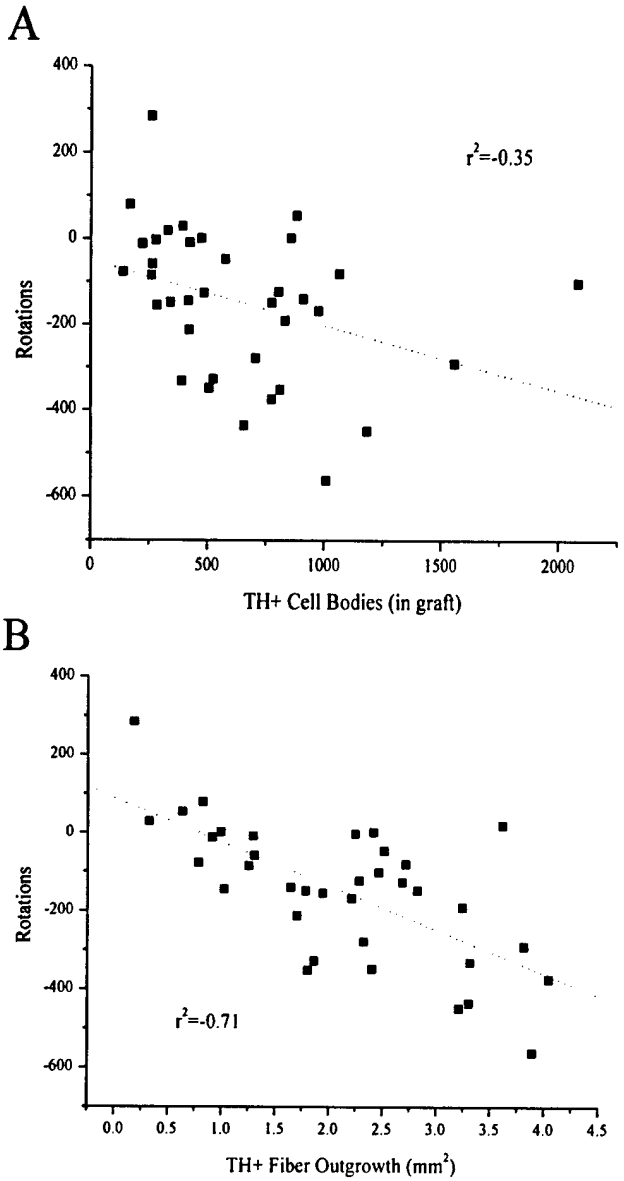

Fig. 5. Correlations between rotational scores, the number of grafted TH+ neurons, and TH+ fiber outgrowth. Scatter plots of individual rotational scores ($n=37$) were plotted as a function of either the number of TH+ neurons within the animal's graft (A) or the area of TH+ fiber outgrowth from the animal's graft (B). A linear regression analysis was performed on each scatter plot (dotted line). The correlation coefficients for plots (A) and (B) are $r^2=-0.35$ and $r^2=-0.71$, respectively. The reversal of amphetamine-induced rotational behavior by dopamine grafts is more tightly correlated to the area of fiber outgrowth than it is to the number of grafted TH+ neurons.

certainly not the only explanation. For example, grafts implanted into the intact striatum would have to compete with existing dopamine fibers in order to establish functional contacts with target neurons in the host tissue. The limited fiber outgrowth from transplants placed into the intact striatum may be restricted to sites where host dopaminergic neurons are disrupted during the implantation process. A previous study also reported anecdotally that fetal dopamine grafts placed into the intact striatum had a more restricted fiber outgrowth pattern than grafts placed into the denervated striatum [16]. Also, the lower survival rate of TH+ cells within the transplants of the −1 Week group may be directly related to the inability of transplanted neurons to successfully innervate the host tissue. During normal development of the nigrostriatal pathway, midbrain dopamine neurons undergo several stages of programmed cell death that might be a consequence of many immature neurons competing to establish functional contacts with a limited number of targets [19]. Moreover, implanting immature dopamine neurons into a dopamine-rich environment may be detrimental to their survival based upon evidence that dopamine itself may induce apoptosis in developing neurons [40]. Therefore the decreased survival of transplanted neurons in the −1 Week group cannot be entirely explained by the neurotrophic environment of the host brain at the site of implantation. Indeed, results from studies performed in our laboratory and others have shown that the intact striatum maintains expression of several dopaminergic neurotrophic factors in adult brain [33,36,39].

While the area of TH+ fiber outgrowth from grafts was significantly greater for the Week 0 or 4 Weeks groups when compared to the −1 Week group, we observed that the number of TH+ neurons in grafts for the Week 0 or 4 Weeks group was slightly higher but not significantly greater than the number of TH+ neurons in grafts for the −1 Week group. On the other hand, both the number of TH+ neurons and the area of fiber outgrowth were significantly greater for the 1 Week group when statistically compared to the −1 Week group. This suggests that a dynamic change in the neurotrophic environment of the denervated striatum may be occurring during the 1st month after the lesion and/or the 1st month after grafting. Our previous studies have demonstrated that after 1–2 weeks following a 6-OHDA lesion, both survival and outgrowth factors may be up-regulated in the denervated striatum and thus provide an environment that supports the survival and functional outgrowth of grafted neurons. At 4 weeks after the lesion, however, survival factors within the denervated striatum may decline whereas outgrowth factors remain elevated. Interestingly, we observe in young lesioned rats an elevation of BDNF levels in the denervated striatum 4 weeks after a nigrostriatal pathway lesion [32]. As already mentioned, BDNF may have properties of a target-derived neurotrophic factor that stimulates fiber outgrowth more than it does as a survival factor for fetal dopamine grafts. Interestingly, fiber outgrowth in the 12 Weeks group is significantly less than that observed in the 1 Week group, and this corresponds to the same post-lesion period when striatal BDNF is not significantly elevated in the striatum of rats with lesions only. Likewise, we observe that GDNF is significantly elevated in the denervated striatum 2 weeks after a 6-OHDA lesion, but this elevation may only be transient because we do not observe a significant differ-

ence in GDNF protein between the intact and lesioned striatum during the 12th post-lesion week. Glial cell line-derived neurotrophic factor is known to be a potent survival factor for dopamine neurons in vitro and in vivo [1,4,8,11,13,21–23,26,28,31]. If striatal GDNF levels are increased only transiently during the first 2 weeks following a 6-OHDA lesion, then this may provide a partial explanation why the number of TH+ neurons was significantly greater in the 1 Week group than in the −1 Week group, and why the comparison of TH+ neurons for the −1 Week, Week 0, and 4 Weeks groups did not yield a significant difference.

We also observe that graft-mediated reduction of amphetamine-induced rotational behavior was more tightly correlated with the degree of fiber outgrowth from the graft than it was with the actual number of surviving TH+ neurons within the graft. Grafted rats showing the largest areas of fiber outgrowth also showed the highest degree of functional overcompensation when tested with amphetamine. The phenomenon of overcompensation in amphetamine-induced rotational behavioral has been ascribed to grafted fibers forming contacts with corticostriatal fibers because the abolition of corticostriatal afferents also blocks this over-compensatory response [6]. Another explanation for this over-compensatory response to amphetamine may be related to the status of striatal dopamine receptors or to an inefficient reuptake of dopamine by grafted neurons; it still remains uncertain whether striatal dopamine receptors are fully normalized by the grafts. From a therapeutic standpoint, it remains to be determined whether or not fiber contacts made between the graft and host neurons are aberrant or functionally useful. Nevertheless, the extent of fiber outgrowth from grafts may be a better predictor of graft-mediated reduction of amphetamine-induced rotational behavior than the actual number of surviving grafted TH+ neurons. This is consistent with the earlier report that behavioral recovery is correlated with the extent of graft fiber reinnervation of the host brain [2,10,29].

It would be interesting to observe whether or not dopamine grafts show enhanced survival and function in the lesioned striatum of aged rats. Studies from our laboratory indicate that protein levels of at least two neurotrophic factors, BDNF and GDNF, are greater in lesioned striatum than in the intact striatum in young rats whereas there are no significant differences in either BDNF or GDNF protein levels between the lesioned and intact striata of aged rats [32,33]. The results of these studies suggest that the neurotrophic environment of the denervated striatum of aged rats may be comparable to the intact striatum of young or old rats. This is intriguing because Collier et al. [9] recently reported that dopamine grafts showing improved transplant function in young animals were virtually without effect in aged rats; this study also reported impaired morphological development of grafts and, in particular, a reduction of fiber outgrowth from grafts placed into aged denervated striatum. In the present study we observed a significant reduction of fiber outgrowth from grafts placed into the intact striatum. The significance of these studies may be more fully appreciated in light of the results from a recent clinical trial using dopamine neuron implants in Parkinson's patients that concluded that patients under 60 years of age exhibited statistically significant clinical benefits from transplants while patients older than 60 years of age did not [15]. It is conceivable that while dopamine grafts placed into elderly Parkinson's patients show evidence of graft survival in terms of PET scan studies, the functional outgrowth of these grafts may be impaired due to an impoverished neurotrophic environment. This may be one explanation why younger Parkinson's patients benefit more from dopamine grafts than older patients.

In conclusion, neurotoxic lesions of the nigrostriatal pathway may induce a transient increase of neurotrophic activity that is initially beneficial to the survival and function of dopamine grafts. In addition, the length of time between the lesion and the grafting procedure may have a direct effect on the success of grafted fetal dopamine neurons in terms of their survival and functional reinnervation of the host. These effects may be directly related to the reports from other studies that have provided evidence that neurotoxic lesions of the nigrostriatal pathway induce a compensatory increase of neurotrophic activity in the denervated striatum.

Acknowledgements

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Research report

Differential expression of GDNF, BDNF, and NT-3 in the aging nigrostriatal system following a neurotoxic lesion

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Abstract

Protein levels for brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and glial cell line-derived neurotrophic factor (GDNF) were measured in the striatum and ventral midbrain of young and aged Brown Norway/F344 F1 (F344BNF$_1$) hybrid rats following a unilateral 6-hydroxydopamine (6-OHDA) lesion of the nigrostriatal pathway. At 2 weeks post-lesion, protein levels of BDNF and GDNF were higher in the denervated striatum when compared to the intact striatum for young (4–5 months old) but not old (31–33 months old) rats. Interestingly, in old rats BDNF protein in the denervated striatum was significantly lower than that measured in the intact striatum. At the same time point BDNF protein levels in the ventral midbrain were higher on the lesioned versus intact side for both young and old rats while no significant side differences were detected for GDNF protein in the ventral midbrain of young or old rats. No significant differences in NT-3 protein levels were detected between the lesioned and intact sides for striatal or ventral midbrain regions in either young or old brain. While no significant age effects were detected for BDNF or NT-3 protein, young rats showed higher GDNF protein levels in both the striatum (lesioned or intact) and ventral midbrain (lesioned or intact) than old rats. These data show that two endogenous neurotrophic factors, BDNF and GDNF, are differentially affected by a 6-OHDA lesion in the aging nigrostriatal system with young brain showing a significant compensatory increase of these two factors in the denervated striatum while no compensatory increase is observed in aged brain.

Theme: Development and regeneration

Topic: Neurotrophic factors: expression and regulation

Keywords: Neurotrophin-3; Brain-derived neurotrophic factor; Glial cell line-derived neurotrophic factor; Neurotrophic factor; Parkinson's disease; Brown/Norway F334 F$_1$ hybrid rats; 6-hydroxydopamine; Dopamine

1. Introduction

Animal models of Parkinson's disease are typically produced by lesioning the nigrostriatal pathway with various neurotoxins, e.g., 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) [18,37]. These lesions consequentially produce a hallmark symptom of Parkinson's disease, e.g., a loss of midbrain dopamine neurons. There is accumulating evidence that these lesions may also induced a compensatory cascade of neurotrophic activity within the nigrostriatal system as a physiologic response to the loss of dopamine neurons in young adult animals. This effect can be discerned from the results of the following studies. First, while extracts taken from the normal striatum enhance the survival and growth of cultured dopamine neurons [7,34], extracts taken from the lesioned striatum appear to provide more potent neurotrophic support. For example, striatal extracts taken from the lesioned striatum of young adult rats improve the survival of cultured dopamine neurons better than extracts taken from the normal striatum [3,21]. This effect has been extended to human dopamine neurons: cultures incubated with extracts from the caudate/putamen of patients with Parkinson's disease contained more tyrosine hydroxylase immunoreactive neurons than extracts obtained from aged controls [4]. Hida et al. demonstrated that striatal extracts taken from the lesioned striatum have stronger effects to hasten the differentiation of PC12D cells, promote neurite

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outgrowth, cell enlargement, and expression of voltage-dependent cation channels when compared to the effects of extracts taken from the normal striatum [13]. More recently, specific neurotrophic factors native to the striatum have been shown to increase following a neurotoxic lesion of the nigrostriatal pathway. In young adult rats with unilateral 6-OHDA lesions, brain-derived neurotrophic factor (BDNF) protein levels are significantly elevated in the lesioned striatum and lesioned ventral midbrain when compared to BDNF protein levels in the same brain regions on the intact side [41,43].

Recent studies have provided evidence that the increase in neurotrophic activity in the denervated striatum is not consistent across the age of the lesioned animals. Ling et al. recently reported that the trophic activity of tissue extracts taken from the lesioned striatum of rats is inversely correlated to the age of the rat [20]. Similarly, Kaseloo et al. reported that striatal extracts taken from the injured striatum of aged rats possessed a diminished capacity for inducing neurite outgrowth in cultures containing a dopamine-producing neuroblastoma cell line [16]. Our recent study showed a compensatory increase of BDNF in the lesioned striatum 4 weeks after the lesion in young but not old rats [41]. The results of these studies suggest young and old brain may respond differently to neurodegenerative events: old brain shows a diminished capacity to elicit compensatory neurotrophic mechanisms. This area of research has been relatively overlooked in animal models of Parkinson's disease.

The purpose of this study was to further characterize how protein levels for three different neurotrophic factors [BDNF, neurotrophin-3 (NT-3), and glial cell line-derived neurotrophic factor (GDNF)] are affected by a neurotoxic lesion of the nigrostriatal pathway in both young and aged rats.

2. Material and methods

2.1. Animals

Young (4–5-month-old, $n=21$) and old (31–33-month-old, $n=14$) male Brown Norway/F344 F1 hybrid rats ($F344BNF_1$) rats were obtained from the NIA Aging Colony. Animals were housed in environmentally regulated rooms and had free access to food and water for the duration of the study. All animal procedures were conducted in strict compliance with approved institutional protocols, and in accordance with the provisions for animal care and use described in the 'Guide for the Care and Use of Laboratory Animals' (NIH publication No. 86-23, NIH, 1985).

2.2. 6-Hydroxydopamine lesions

Male $F344BNF_1$ rats in each age group were given unilateral 6-hydroxydopamine (6-OHDA) lesions of the nigrostriatal pathway; 6-OHDA (Sigma) was dissolved in 0.9% saline (containing 0.2% ascorbic acid) at a concentration of 2.0 μg/μl and stereotactically injected into the nigrostriatal pathway of anesthetized rats at a rate of 1.0 μl/min for 3 min. Each rat received two injections of 6-OHDA: one in the vicinity of the medial forebrain bundle (AP −4.3, ML 1.2, DV −7.5) and the other in the rostral pars compacta of the substantia nigra (AP −4.8, ML 1.5, DV −7.5); all coordinates reported in this study represent millimeter adjustments from bregma (AP, ML) and below the dural surface (DV) with the top of the skull in a flat position. This technique routinely produces complete lesions of A9 and A10 midbrain regions, and near-complete denervation of dopaminergic fibers innervating the ipsilateral striatum [33].

2.3. Quantification of neurotrophic factors by an enzyme-linked immunosorbent assay (ELISA)

Animals were euthanized 2 weeks after the 6-OHDA lesion. Brains were removed, the striatal and substantia nigra/ventral tegmental area (SN/VTA) brain regions were dissected on ice, and the samples were then stored at −80°C. Subsequently, each tissue sample was homogenized in 400-μl volumes of homogenate buffer [400 mM NaCl, 0.1% Triton-X, 2.0 mM EDTA, 0.1 mM benzethonium chloride, 2.0 mM benzamidine, 0.1 mM PMSF, Aprotinin (9.7 TIU/ml), 0.5% BSA, 0.1 M phosphate buffer, pH=7.4]. The homogenate was centrifuged for 10 min at 10 000×g at 4°C. The homogenate was divided into 100-μl duplicate samples and neurotrophic factor content was determined using an antibody sandwich format: extracted neurotrophic factors from each sample were captured with a monoclonal antibody against BDNF, GDNF, or NT-3; the captured BDNF was then bound to a second, specific, polyclonal antibody (pAb) against BDNF, GDNF, or NT-3. After washing, the amount of specifically bound pAb was detected using a species-specific anti-IgY antibody conjugated to horseradish peroxidase (HRP) as a tertiary reactant. Unbound conjugate was removed by washing and, following an incubation period with a chromogenic substrate, the color change was measured in a microplate reader (450 nm). The amount of neurotrophic factor was proportional to the color change generated in an oxidation–reduction reaction.; the Promega E_{max}™ ImmunoAssay System was used for the detection of all three neurotrophic factors. The reliability of the neurotrophic factor measures ranged from 97 to 99% based upon regression analysis.

2.4. Statistical analysis

Comparison of side differences (lesion vs. intact) for neurotrophic factor protein levels were made using a paired t-test for each age group. Analysis of variance

(ANOVA) was used to analyze age differences in the data. The alpha level was set to 0.05.

3. Results

3.1. NT-3

There was no significant effect of age on NT-3 protein levels in the intact or lesioned striatum [$F(1,16)=0.27$, $P>0.05$], or in the lesioned or intact ventral midbrain [$F(1,16)=0.89$, $P>0.05$]. Neurotrophin-3 levels in the lesioned striatum were not significantly different from the intact striatum for either young ($P=0.70$) or old rats ($P=0.63$). Similarly, no significant differences in NT-3 were detected between the lesioned and intact ventral midbrain for young ($P=0.51$) or old rats ($P=0.14$). These data are summarized in Fig. 1.

3.2. BDNF

Fig. 2 shows a comparison of mean BDNF values in the lesioned or intact striatum of young or old rats. Two weeks after the lesion young rats show a higher level of BDNF protein in the lesioned striatum when compared to the intact striatum ($P=0.01$). On the other hand, in old rats BDNF protein levels are significantly lower in the lesioned striatum than in the intact striatum ($P<0.001$). A significant effect of age on striatal BDNF levels [$F(1,47)=6.32$, $P=0.016$] was detected between the lesioned striatum of young and old rats ($P<0.05$) while the effect of age was not significant for the intact striatum ($P>0.05$).

Comparison of BDNF protein levels in the ventral midbrain of young and old rats show significantly higher levels of BDNF protein in the lesioned vs. the intact side for both young ($P=0.01$) and old ($P=0.038$) rats. There

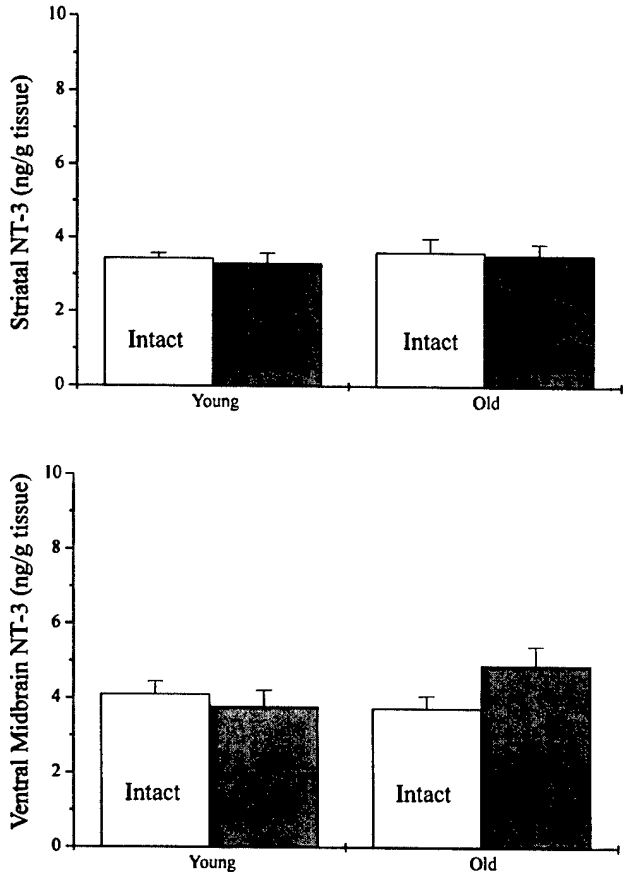

Fig. 1. NT-3 protein levels (ng/g tissue) in the striatum (top) or ventral midbrain (bottom) of young ($n=5$, 4–5-month-old) or old ($n=5$, 31–33-month-old) F344BNF$_1$ rats. Animals were given a unilateral 6-OHDA lesion and sacrificed 2 weeks later. Tissue was dissected from the striatum and ventral midbrain from both the lesioned and intact hemispheres and subjected to ELISA analysis.

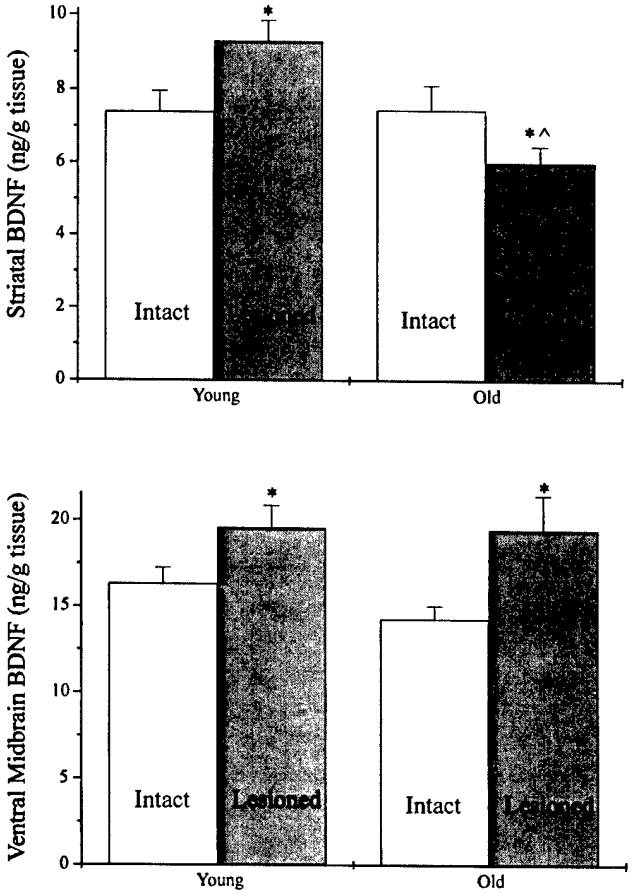

Fig. 2. BDNF protein levels (ng/g tissue) in the striatum (top) or ventral midbrain (bottom) of young ($n=16$, 4–5-month-old) or old ($n=9$, 31–33-month-old) F344BNF$_1$ rats. Animals were given a unilateral 6-OHDA lesion and sacrificed 2 weeks later. Tissue was dissected from the striatum and ventral midbrain from both the lesioned and intact hemispheres and subjected to ELISA analysis. *$P<0.05$, intact vs. lesioned; ^$P<0.05$, young vs. old.

was no significant effect of age on BDNF protein levels in ventral midbrain on either side [$F(1,47)=0.57$, $P=0.452$].

3.3. GDNF

The results for GDNF analysis are shown in Fig. 3. Two weeks after the lesion young rats showed higher GDNF protein levels in the lesioned striatum than in the intact striatum ($P<0.001$). In old rats GDNF protein levels in the lesioned and intact striatum were not statistically different from one another ($P=0.98$). Analysis of variance revealed a significant effect of age on GDNF protein levels in the striatum [$F(1,49)=28.14$, $P<0.001$]. The lesioned striatum of young rats contained higher levels of GDNF protein than the lesioned striatum of old rats ($P<0.05$), and the intact striatum of young rats contained higher levels of GDNF protein than the intact striatum of old rats ($P<0.05$).

We observed only a slight but non-significant increase of GDNF protein on the lesioned side in the ventral midbrain of young rats ($P=0.41$). Similarly, midbrain levels of GDNF in the lesioned and intact sides were not significantly different from one another ($P=0.80$). The effect of age on ventral midbrain GDNF levels was significant [$F(1,49)=21.45$, $P<0.001$]. The lesioned ventral midbrain of young rats contained higher levels of GDNF protein than the lesioned ventral midbrain of old rats ($P<0.05$), and the intact ventral midbrain of young rats contained higher levels of GDNF protein than the intact ventral midbrain of old rats ($P<0.05$).

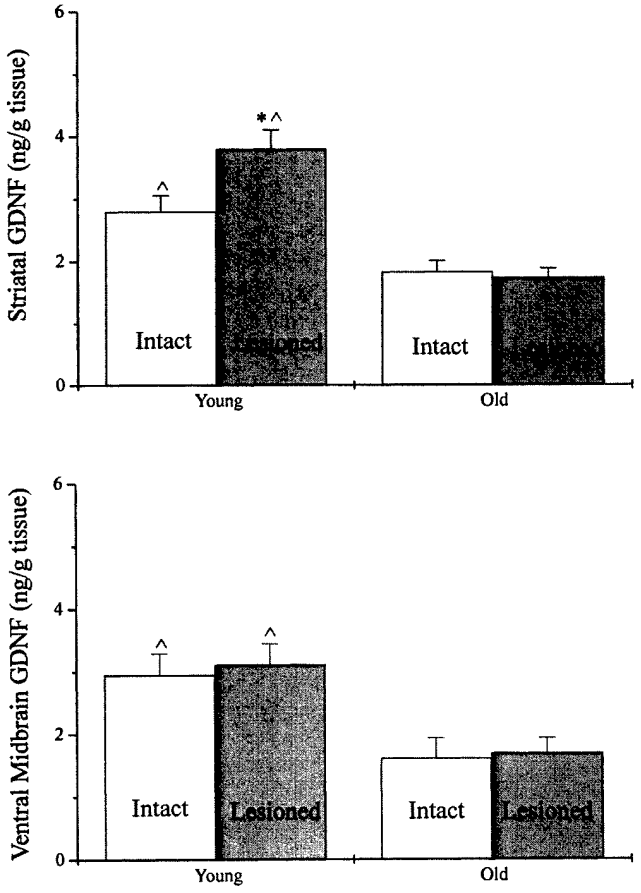

Fig. 3. GDNF protein levels (ng/g tissue) in the striatum (top) or ventral midbrain (bottom) of young ($n=16$, 4–5-month-old) or old ($n=9$, 31–33-month-old) F344BNF$_1$ rats. Animals were given a unilateral 6-OHDA lesion and sacrificed 2 weeks later. Tissue was dissected from the striatum and ventral midbrain from both the lesioned and intact hemispheres and subjected to ELISA analysis. *$P<0.05$, intact vs. lesioned; ^$P<0.05$, young vs. old.

4. Discussion

The results of this study provide evidence that the expression of three different neurotrophic factors within the mesostriatal system are differentially affected by a neurotoxic lesion of the nigrostriatal pathway during aging. In young rats the expression of two neurotrophic factors, BDNF and GDNF, increase within the denervated 2 weeks following a nigrostriatal lesion while NT-3 protein levels in the denervated striatum did not change significantly. In aged rats protein expression of BDNF was significantly reduced in the denervated striatum while GDNF and NT-3 did not change significantly. Protein levels of BDNF in the lesioned ventral midbrain were significantly higher than those observed in the intact ventral midbrain in both young and aged rats. Glial cell line-derived neurotrophic factor was the only one of the three proteins studied to show an age-related reduction in both the lesioned and intact mesostriatal system of F344BNF$_1$ rats.

Glial cell line-derived neurotrophic factor is a distant member of the TGF-β family of neurotrophic factors and is expressed in the substantia nigra and striatum, as well as other brain regions, in both the developing and adult brain of rats and humans [25,26,31]. The functional receptor for GDNF is a two-component receptor complex that consists of a ligand binding GDNF family receptor, GDNFR-α1 or GDNFR-α2, and the receptor protein kinase ret [10,15,35,36]. In rats, dopamine neurons express both GDNFR-α mRNA and ret mRNA during development and throughout adulthood while only GDNFR-α mRNA is expressed in the ventral striatum during development [22]. The ret protein has been identified immunohistochemically to be on dopamine neurons in adult rat brain [22]. Thus the functional receptor of GDNF appears to be present in dopamine neurons throughout the lifetime of rats. Injury to dopamine neurons or the striatum can elicit changes in the expression of GDNF or its receptor. For instance, while GDNF mRNA expression is not observed in the striatum of normal adult rats [32], its expression in the striatum can

be induced by status epilepticus in motor and limbic brain regions [27]. In mice, MPTP treatment does not change the expression of GDNF mRNA in the denervated striatum [14] while mechanical injury to the striatum elicits an increased expression of GDNF mRNA [19]. Ischemic brain injury via occlusion of the middle cerebral artery can induce GDNFR-α1 and ret in the striatum [17]. In the present study we observe that GDNF protein is increased in the denervated striatum of young rats. This increased expression of GDNF protein, GDNF mRNA, and GDNFR mRNA in the denervated striatum may be a compensatory neurotrophic response to the loss of striatal afferents and/or direct injury to the striatum.

Previous studies have established that the neurotrophins BDNF and NT-3, along with their receptors [trkB and trkC], are expressed within the mesostriatal system during development and throughout adulthood [11,24,29,30]. The profuse expression of these neurotrophins and their receptors in the ventral midbrain during development suggests that these two neurotrophins may play an important role for the differentiation, maturation, and target innervation of dopamine neurons. The sustained expression of these neurotrophins and their receptors in adult brain suggests a role for the maintenance and repair of the mesostriatal system throughout the lifetime of the organism. Injury to the mesostriatal system alters the expression of neurotrophins and neurotrophin receptors in young adult rodents. For instance, transection of the medial forebrain bundle induces an up-regulation of trkB protein in the ipsilateral striatum [8]. The expression of the full-length form of trkB mRNA in the denervated striatum is up-regulated at 2 weeks [23] and 8 weeks [42] after the nigrostriatal pathway is neurotoxically lesioned with 6-OHDA. Following a mechanical injury to mouse striatum, the expression of BDNF mRNA and the truncated, but not full-length, form trkB mRNA are increased in the injured striatum [39]. The results of the present study along with those reported by Zhou et al. [43] show an increase of BDNF in the denervated striatum in young adult rats. Taken together, the results of the aforementioned studies provide convincing evidence that the expression of BDNF and trkB receptor increase as a consequence of striatal injury or a neurotoxic lesion of the nigrostriatal pathway of young adult rodents. On the other hand, we did not observe a change in striatal NT-3 protein levels 2 weeks following a 6-OHDA lesion in either young or old rats, nor does the expression of trkC mRNA change significantly in the denervated striatum of young adult rats 2 weeks after a 6-OHDA lesion [23]; it is noteworthy that unlike the increase of trkB mRNA expression 8 weeks after a 6-OHDA lesion, the expression of trkC mRNA is actually decreased in the denervated striatum of young rats [42]. In this study we observe the two neurotrophins, BDNF and NT-3, are differentially expressed in the denervated striatum of young adult rats in response to a lesion of the nigrostriatal pathway. In aged rats, however, we provide evidence that at least three neurotrophic factors [BDNF, NT-3, or GDNF] do not show a compensatory increase following a 6-OHDA lesion of the nigrostriatal pathway.

The lack of a compensatory increase in BDNF or GDNF within the lesioned striatum of aged rats is consistent with other neurotrophic factors in other denervated brain regions. For example, following a medial septal lesion only young rats demonstrated significant increases in sympathetic sprouting and NGF-like activity in the hippocampus [28]; this suggests that the age-related deficit in sympathetic sprouting may result from an attenuated neurotrophic response to hippocampal denervation, similar to what we observe in the denervated striatum of old rats. It still remains unclear why compensatory neurotrophic mechanisms may diminish with age.

Interestingly, we observed better survival, fiber outgrowth, and functional reinnervation for fetal ventral mesencephalic tissue transplants when the tissue is implanted 1 or 4 weeks after a 6-OHDA lesion rather than 1 week before the 6-OHDA lesion [40]. Not surprisingly, the post-lesion period when transplant development is robust also coincides with the post-lesion period when at least two neurotrophic, BDNF and GDNF, are increased in the denervated striatum. The expression of other neurotrophic factors, e.g., bFGF [5], are increased in the denervated striatum immediately following a nigrostriatal pathway lesion. A critical period for the survival of transplanted dopamine neurons occurs during the first 4 days immediately following implantation [9]. During this critical period, fetal neurons implanted into the intact striatum 1 week prior to a 6-OHDA lesion would not be exposed to the same enriched neurotrophic environment as those implanted after the lesion. The results of this study strongly suggest that the striatal environment of the intact striatum may not be as conducive to the survival, fiber outgrowth, and function of transplants as is the lesioned striatum. This is consistent with the results of the present study and with the results of previous studies that demonstrated prior injury to the striatum improves the survival of fetal dopamine implants [1,2]. Up-regulation of neurotrophic activity in the injured or denervated striatum of young animals may actually be beneficial to the survival and functional reinnervation of implanted donor cells. In old rats, however, we did not observe an increase of BDNF or GDNF protein levels in the denervated striatum. This may be a significant finding in terms of the success that fetal cell implants may have in aged brain. The recent study completed by Collier et al. [6] provides compelling evidence that transplants in the aged brain show a poorer survival rate and less functional compensation than transplants into young brain; therefore the age of the transplant recipient may be an important determinant for the survival and/or functional effects of fetal mesencephalic transplants. Furthermore, a recently completed clinical trial using dopamine neuron implants in Parkinson's patients concluded that patients under 60 years of age exhibited

statistically significant clinical benefits from transplants while patients older than 60 years of age did not [12]. The results of the present study provide initial evidence that the denervated striatum of young rats may become neurotrophically enriched following a degenerative lesion of the nigrostriatal pathway and thus provide a more nurturing environment for transplant development than in aged brain.

In the present study we observed significantly lower levels of BDNF in the lesioned striatum than in the intact striatum of old rats at the 2-week post-lesion time point. In a previous study we reported that BDNF protein levels in the lesioned and intact striatum of aged rats were not significantly different from one another at the four week post-lesion time point [41]. The results of the present study are not entirely inconsistent with our previous report, however. Previously we reported that at the four week post-lesion time point, the most severely lesioned old rats tended to show a greater reduction of BDNF in the denervated striatum than old rats with less severe lesions.

While previous studies have shown a reduction in BDNF mRNA labeling within the substantia nigra following a lesion of the dopamine cell bodies [29,30,38], we observe an increase of BDNF protein in the lesioned ventral midbrain of both young and old rats. Seroogy et al. [29,30] report approximately 20% of BDNF mRNA labeling in the ventral mesencephalon occurs in non-dopaminergic cell bodies, and Venero et al. [38] report a continued expression of BDNF mRNA labeling within the ventral tegmental area and pars lateralis of the substantia nigra following a 6-OHDA lesion. Taken together, these data provide evidence that BDNF mRNA is localized to dopaminergic and non-dopaminergic cell bodies within the ventral mesencephalon. The increase of BDNF protein within the lesioned ventral midbrain may result from a local compensatory reaction to the lesion by non-dopaminergic neurons and a concomitant accumulation of BDNF that might occur after dopamine neurons, which normally bind and take up BDNF, are lost as a result of the lesion. The increase in BDNF protein in the lesioned ventral midbrain of young animals are consistent with the increase of BDNF content in the lesioned substantia nigra observed 2 weeks [43] and 4 weeks [41] after the lesion.

Because we were unable to assess the degree of the 6-OHDA lesion prior to obtaining our samples, it is possible that our final analysis of the data included samples taken from animals with incomplete or poor lesions. The short interval between the time the animals were lesioned and the time the animals were sacrificed did not allow us to use conventional tests to accurately assess lesion severity, e.g., amphetamine- or apomorphine-induced rotational behavior. In addition, no tissue samples were available for the determination of dopamine content in either the substantia nigra or striatum because all samples were used for ELISA analysis. This may be one explanation why the difference in BDNF protein levels between the lesioned and intact striata at 2 weeks post-lesion was not as great as that observed 4 weeks post-lesion [41]; at 4 weeks post-lesion, animals with no evidence of a lesion were excluded from the study. Another explanation for this phenomenon is that BDNF protein levels in the denervated striatum increase progressively following a nigrostriatal pathway lesion. In order to determine whether the increase of lesion-induced neurotrophic activity is progressive, transient, or both, the time course of this phenomenon needs to be more fully characterized.

In conclusion, neurotoxic lesion of the nigrostriatal pathway affects the expression of several specific neurotrophic factors differentially, and the expression is also dependent upon the age of the animal. Both BDNF and GDNF protein levels in the lesioned striatum are increased 2 weeks following a 6-OHDA lesion whereas these same two neurotrophic factors do not show a compensatory increase in the lesioned striatum of old rats. The expression of GDNF shows an age-related decline in both the lesioned and intact striatum. The results of this study provide evidence that young animals show an enhanced neurotrophic response to a neurotoxic lesion that is not observed in older animals. The differential expression of these neurotrophic factors may have a direct effect on the success of therapies which use cellular implants to correct neurodegenerative disorders, particularly if the cellular implants are dependent upon neurotrophic factors for differentiation, survival, and the maintenance of function.

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BRIEF COMMUNICATION

Lesion-Induced Increase of BDNF Is Greater in the Striatum of Young versus Old Rat Brain

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Young (4–5 month old) and old (32–34 month old) Brown Norway/F344 hybrid rats were given unilateral 6-OHDA lesions of the nigrostriatal pathway. Four weeks later tissue from the lesioned or intact striatum or ventral midbrain was dissected and analyzed for brain-derived neurotrophic factor (BDNF) protein levels using an enzyme-linked immunosorbent assay. BDNF protein content was greater in the lesioned striatum than in the intact striatum for all young rats, and the increased BDNF content in the lesioned striatum of young rats was directly correlated with severity of lesion as determined by rotational scores. BDNF content in the lesioned striatum increased in less than half of the old rats and was not significantly different than BDNF content in the intact striatum. BDNF content in the lesioned substantia nigra/ventral tegmental area (SN/VTA) was greater than BDNF content in the intact SN/VTA for both young and old rats. These data suggest that an age-related difference in activity of at least one neurotrophic factor, BDNF, occur within the denervated striatum following a neurotoxic lesion of the nigrostriatal pathway. © 2000 Academic Press

Key Words: **aging; dopamine; nigrostriatal; Parkinson's disease; 6-hydroxydopamine; neurotrophic factor; Brown Norway/Fischer 344 F1 hybrid rats.**

Evidence from culture and neural transplantation studies suggest that there may an age-related decline in neurotrophic activity within the mesostriatal system. For example, Carvey *et al.* noted that the compensatory increase in striatal trophic activity following a 6-OHDA lesion is lower in aged than in young rats (1). Similarly, Kaseloo and coworkers (5) reported that striatal extracts taken from the injured striatum of aged rats possessed a diminished capacity for inducing neurite outgrowth in cultures containing SH-SY5Y cell line [dopamine-producing neuroblastoma cell line]. In aged rats with long-term 6-OHDA lesions, transplants show poor survival and function unless neurotrophic supplements, e.g., Schwann cells, are co-grafted with transplants (2–4). The results of these studies suggest that the compensatory neurotrophic mechanisms that occur following a degenerative lesion may decline with age. This study examined protein levels of one neurotrophin, brain-derived neurotrophic factor (BDNF), that is known to exert neurotrophic support for dopaminergic neurons in both *in vitro* and *in vivo* studies (13). Using an enzyme-linked immunosorbent assay (ELISA), we measured BDNF protein in the intact and denervated striatum following a unilateral 6-hydroxydopamine (6-OHDA) lesion of the nigrostriatal pathway in both young and aged brain.

Young (4–5 month old, $n = 9$) and old (32–34 month old, $n = 11$) Brown Norway/F344 F1 hybrid rats ($F344BNF_1$) were obtained from the NIA Aging Colony. All rats from each age group were given unilateral 6-hydroxydopamine (6-OHDA) lesions of the nigrostriatal pathway; 6-OHDA (Sigma) was dissolved in 0.9% saline (containing 0.2% ascorbic acid) at a concentration of 3.0 μg/μl and stereotactically injected into the nigrostriatal pathway of anesthetized rats at a rate of 1.0 μl/min for 2 min. Each rat received two injections of 6-OHDA: one in the vicinity of the medial forebrain bundle (AP −4.3, ML 1.2, DV −7.5) and the other in the rostral pars compacta of the substantia nigra (AP −4.8, ML 1.5, DV −7.5); all coordinates reported in this study represent millimeter adjustments from bregma (AP, ML) and below the dural surface (DV) with the top of the skull in a flat position. Animals were tested for amphetamine [5.0 mg/kg, i.p.] rotational behavior 3 weeks after the lesion; partial lesions were acceptable in this study and only animals showing <150 net ipsiversive rotations over a 90-min testing period were excluded from group analysis. Mean rotational scores for young rats ($n = 9$) were 532.9 ± 75.3 rotations/90 min and 544.7 ± 51.8 rotations/90 min for old rats ($n = 11$). Animals were euthanatized 4 weeks after the 6-OHDA lesion. Brains were removed, the striatal and substantia nigra/ventral tegmental area (SN/VTA) samples were dissected on ice, and the samples were

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stored at −80°C. Subsequently, each tissue sample was homogenized in 400-μl volumes of homogenate buffer [400 mM NaCl, 0.1% Triton-X, 2.0 mM EDTA, 0.1 mM benzethonium chloride, 2.0 mM benzamidine, 0.1 mM PMSF, Aprotinin (9.7 TIU/ml), 0.5% BSA, 0.1 M phosphate buffer, pH 7.4]. The homogenate was centrifuged for 10 min at 10,000 *g* at 4°C. The homogenate was divided into 100-μl duplicate samples and BDNF content was determined using an antibody sandwich format: extracted BDNF from each sample was captured with a BDNF monoclonal antibody, the captured BDNF was then bound to a second, specific, BDNF polyclonal antibody (pAb). After washing, the amount of specifically bound pAb was detected using a species-specific anti-IgY antibody conjugated to horseradish peroxidase (HRP) as a tertiary reactant. Unbound conjugate was removed by washing and, following an incubation period with a chromogenic substrate, the color change was measured in a microplate reader (450 nm). The amount of BDNF was proportional to the color change generated in an oxidation-reduction reaction (Promega E_{max} ImmunoAssay System). The reliability of the BDNF measures ranged from 97–99% based upon regression analysis.

Figures 1 and 2 shows the results of the ELISA analysis. Data were statistically analyzed using two-way ANOVA with repeated measures. A statistically significant interaction between the variables AGE and STRIATUM (Intact or Lesion) was detected [$F(1, 18) = 4.87$, $P < 0.05$]. BDNF content in the intact striatum was similar for young and aged rats [$P > 0.05$]. BDNF content was greater in the denervated striatum than it was in the intact striatum for all nine young rats [$P < 0.01$]. There was no significant difference in BDNF content between the intact and denervated striatum of aged rats [$P > 0.05$], and only 5 of the 11 aged rats showed higher BDNF content in the denervated striatum than in the intact striatum. BDNF content in the lesioned SN/VTA was greater than in the intact SN/VTA for both young and old rats (Fig. 2, $P < 0.01$). Correlations between rotational scores and the increase in BDNF content within the denervated stria-

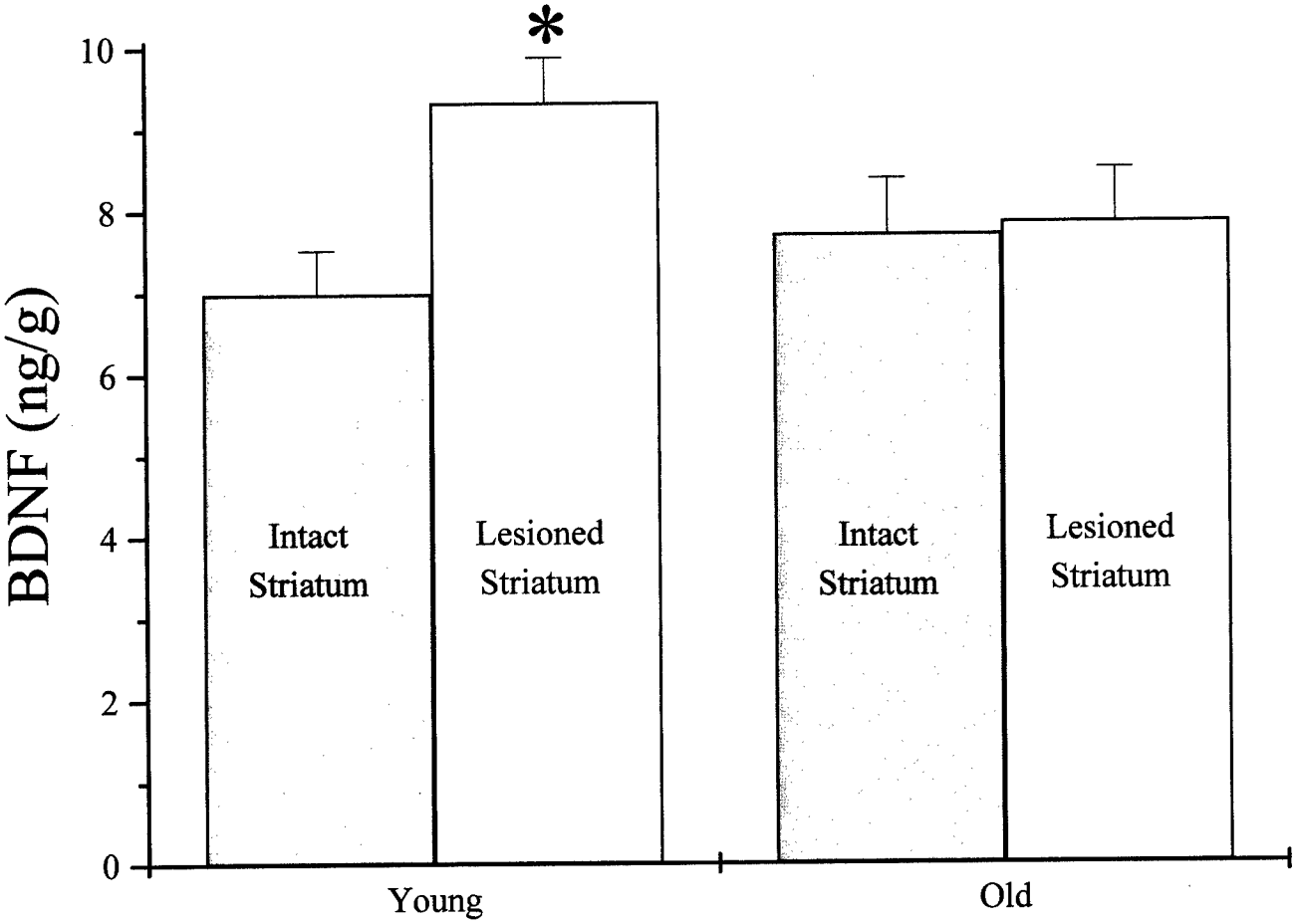

FIG. 1. BDNF protein measured in tissue dissected from the lesioned or intact striatum of young (4–5 month old, $n = 9$) or old (32–34 month old, $n = 11$) F344BNF$_1$ rats. Tissue was dissected 4 weeks following a unilateral 6-OHDA lesion. Error bars, S.E.M. *$P < 0.05$ versus young intact, old intact, and old lesioned.

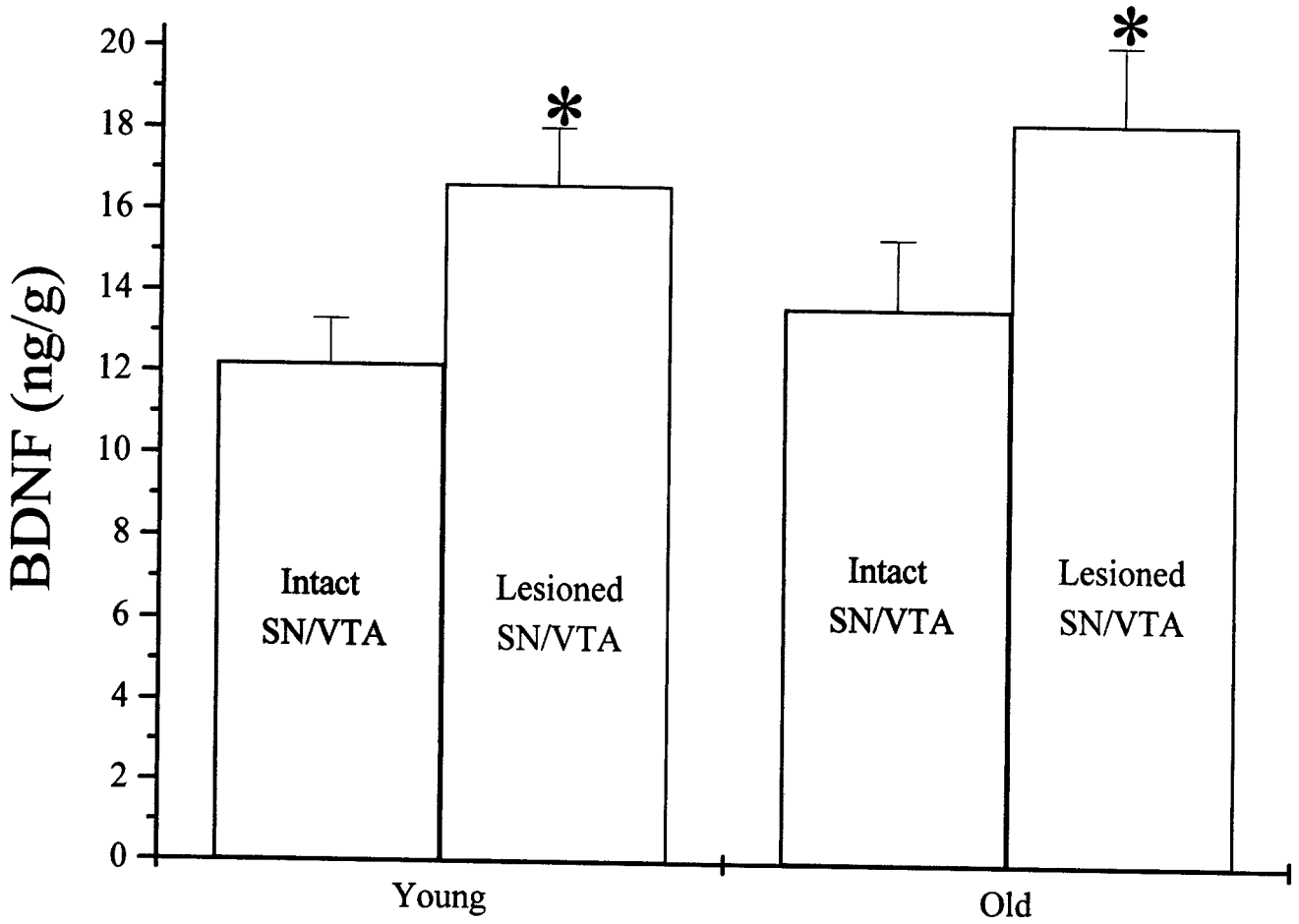

FIG. 2. BDNF protein measured in tissue dissected from the lesioned or intact substantia nigra/ventral tegmental area (SN/VTA) of young (4–5 months old, $n = 9$) or old (32–34 months old, $n = 11$) F344BNF$_1$ rats. Tissue was dissected 4 weeks following a unilateral 6-OHDA lesion. Error bars, S.E.M. *$P < 0.05$ versus intact.

tum for each age group are shown in Fig. 3. For young animals, rotational scores are positively correlated [$r^2 = 0.76$] with changes in BDNF content within the denervated striatum (Fig. 3). A poorer, negative correlation [$r^2 = -0.34$] exists between rotational scores and changes in BDNF content within the denervated striatum for aged rats (Fig. 3). Animals in both age groups with rotational scores <150 rotations/90 min had similar measures of BDNF in both the intact and lesioned striatum.

Our measured values of BDNF content [6–10 ng/g tissue] in the intact striatum of young adult rats are consistent with those reported in previous studies (6, 14). Zhou *et al.* reported a maximal 1.8-fold increase in striatal BDNF content two weeks after a 6-OHDA lesion that eventually return to normal levels by the 7th postlesion week (14). We report that BDNF content in the denervated striatum of young adult F344BNF$_1$ rats remains significantly elevated by the 4th postlesion week in young adult brain, however, the magnitude of the increase is smaller than that report by Zhou *et al.* (14) during the 2nd postlesion week. We have preliminary evidence that BDNF content is also increased in the denervated striatum of young F344BNF$_1$ rats during the first 2 weeks after a lesion (data not shown). We chose to study the changes in BDNF content during the 4th postlesion week because this is typically the time when transplants of neural tissue or cells are implanted into the denervated striatum in studies using the rat model of Parkinsonism. The results of this study demonstrate that the neurotrophic environment of the denervated striatum may not be the same for young and aged animals. Interestingly, if we use rotational scores as an index of the severity of the lesion and then choose the top five scores for each age group, i.e., animals with >450 rotations/90 min, the age difference in increased BDNF content is even more dramatic: all five young rats show increases in BDNF content within the denervated striatum [mean difference = $+3.1 \pm 0.7$], while only two of five old rats show increased BDNF content in the denervated striatum [mean difference = -1.7 ± 0.9]. Statistical analysis

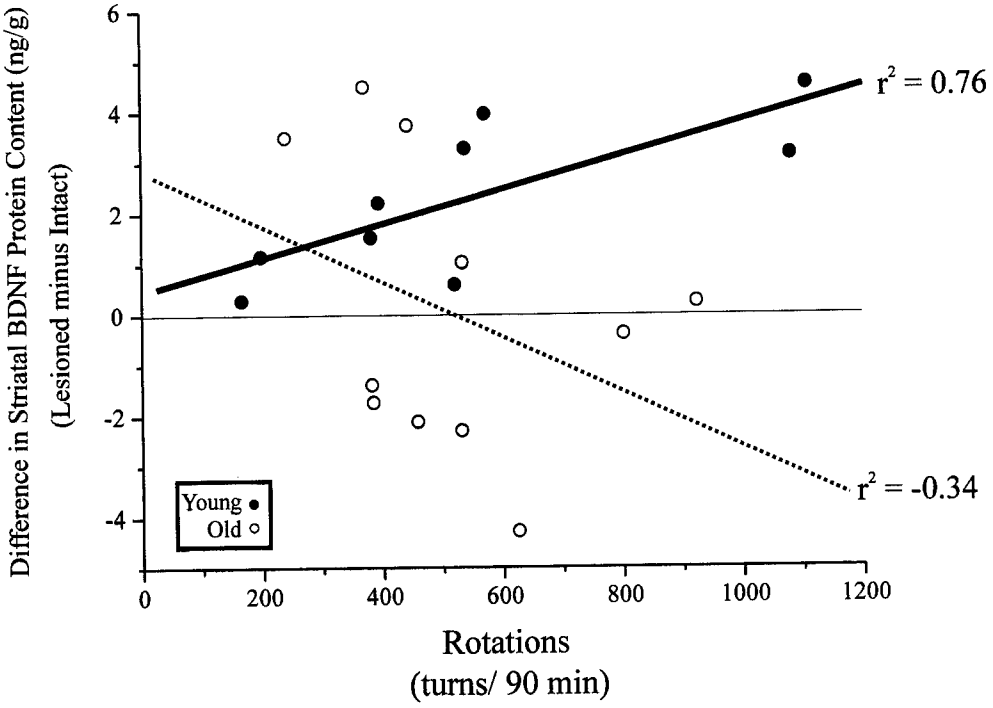

FIG. 3. Correlation between rotational scores and the difference in BDNF protein content between the lesioned and intact striatum of young or old F344BNF$_1$ rats. Change in BDNF protein is defined as the difference between striatal BDNF protein content measured in the lesioned striatum and BDNF protein measured in the intact striatum within the same animal. Therefore a positive change indicates an increase of BDNF content in the lesioned striatum relative to the intact striatum and a negative change indicates a decrease of BDNF content in the lesioned striatum relative to the intact striatum. Black circles, young rats; white circles, old rats. The solid line is the regression line for young rats [$r^2 = 0.76$] and the dotted line is the regression line for old rats [$r^2 = -0.34$].

[*t* test] of these data indicate that the mean difference in BDNF content for the five young rats was significantly greater than the mean difference for the five old rats [$P = 0.003$].

While previous studies have shown a reduction in BDNF mRNA labeling within the substantia nigra following a lesion of the dopamine cell bodies (8, 9, 10), we observe an increase of BDNF protein in the lesioned SN/VTA of both young and old rats. Seroogy *et al.* (8, 9) report approximately 20% of BDNF mRNA labeling in the ventral mesencephalon occurs in nondopaminergic cell bodies, and Venero *et al.* (10) report a continued expression of BDNF mRNA labeling within the VTA and pars lateralis of the substantia nigra following a 6-OHDA lesion. Taken together, these data provide evidence that BDNF mRNA is localized to dopaminergic and nondopaminergic cell bodies within the ventral mesencephalon. The increase of BDNF protein within the lesioned SN/VTA may result from a local compensatory reaction to the lesion by nondopaminergic neurons and a concomitant accumulation of BDNF that might occur after dopamine neurons, which normally bind and take up BDNF, are lost as a result of the lesion. The increase in BDNF protein in the SN/VTA of young animals are consistent with the increase of BDNF content in the lesioned substantia nigra observed by Zhou *et al.* 2 weeks after the lesion (14).

With regard to neural transplantation studies, the results of our study are significant because most of the basic scientific research has been performed in young adult animals with experimental Parkinson's disease while human transplant recipients are typically aged patients with advanced Parkinson's disease. The age disparity between animal and human transplant recipients may prove to be a crucial variable in terms of explaining why animal studies typically yield more promising experimental results than clinical transplant studies. In terms of transplant development, in young rats the increased levels of BDNF may provide beneficial support for the survival and integration of transplanted neural tissue. Previous studies have shown that BDNF improves the function and integration of embryonic mesencephalic neurons implanted into rats with experimental Parkinson's disease (7, 11, 12). Similarly, in order to develop effective strategies for preventing age-related neurodegenerative disorders it will be important to determine whether or not there is a general decline of neurotrophic factor activity or responsiveness to neurotrophic factors in the aging brain.

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